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AB **G-protein-coupled receptors** are a major target for potential therapeutics; yet, a large number of these receptors couple to the Gi pathway, generating signals that are difficult to **detect**. We have combined chimeric G proteins, automated sample handling, and simultaneous 96-well fluorometric imaging to develop a high-throughput assay system for Gi signaling. The chimeric G proteins alter receptor coupling so that signaling can occur through Gq and result in mobilization of intracellular calcium stores. An automated signaling assay device, the fluorometric imaging plate reader (FLIPR), can simultaneously measure this response in real time in 96-well microplates, allowing two people to process more than 10,000 points per day. We used the chimeric G protein/FLIPR system to characterize signaling by the Gi-coupled human opioid receptors. We show that the mu, delta, and kappa opioid receptors and the related nociceptin receptor, ORL1, each couple to Galphaqi5, Galphaqo5, and Galpha16 (Galphaqi5 and Galphaqo5 refer to Galphaq proteins containing the five carboxyl-terminal amino acids from Galphai and Galphao, respectively) and that different receptor/G protein combinations show different levels of maximal activation. We tested 31 opioid ligands for agonist activity at the opioid receptors (124 ligand-receptor combinations); all 31 activated at least one receptor type, and several activated multiple receptors with differing potencies. This high-throughput assay could be useful for dissecting the complex ligand-receptor relationships that are common in nature.

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Chimeric G Proteins Allow a High-Throughput Signaling Assay of G_i-Coupled Receptors

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G-protein-coupled receptors are a major target for potential therapeutics; yet, a large number of these receptors couple to the G_i pathway, generating signals that are difficult to detect. We have combined chimeric G proteins, automated sample handling, and simultaneous 96-well fluorometric imaging to develop a high-throughput assay system for G_i signaling. The chimeric G proteins alter receptor coupling so that signaling can occur through G_q and result in mobilization of intracellular calcium stores. An automated signaling assay device, the fluorometric imaging plate reader (FLIPR), can simultaneously measure this response in real time in 96-well microplates, allowing two people to process more than 10,000 points per day. We used the chimeric G protein/FLIPR system to characterize signaling by the G_i-coupled human opioid receptors. We show that the μ, δ, and κ opioid receptors and the related nociceptin receptor, ORL1, each couple to $G\alpha_{qi5}$, $G\alpha_{qo5}$, and $G\alpha_{16}$ ($G\alpha_{qi5}$ and $G\alpha_{qo5}$ refer to $G\alpha_q$ proteins containing the five carboxyl-terminal amino acids from $G\alpha_i$ and $G\alpha_o$, respectively) and that different receptor/G protein combinations show different levels of maximal activation. We tested 31 opioid ligands for agonist activity at the opioid receptors (124 ligand–receptor combinations); all 31 activated at least one receptor type, and several activated multiple receptors with differing potencies. This high-throughput assay could be useful for dissecting the complex ligand–receptor relationships that are common in nature. © 1999 Academic Press

G-protein-coupled receptors constitute the largest known family of cell-surface receptors (1). Nearly 2000 have been identified (2), and we estimate that more than 100 activate G_i-signaling pathways in response to hormone ligands. G_i signaling is involved in a variety of physiologic processes, including chemotaxis, neurotransmission, proliferation, hormone secretion, and analgesia (3). Scientists in academia and industry have isolated and characterized the genes encoding many medically important G_i-coupled receptors, such as the opioid, serotonin, and dopamine receptors (4). Drugs which target these receptors have found use in the treatment of pain, depression, psychoses, and Parkinson's disease (4). Searching for drugs that target the estimated 100 G_i-coupled receptors presents a major challenge for research.

Several methods exist for measuring signaling by G_i-coupled receptors. Incorporation of radiolabeled GTPγS into receptor-activated G proteins is a popular method, but requires purification of membrane components and handling of radioisotopes, and lacks the intrinsic signal amplification that would occur in a whole cell (5). Because activation of the G_i pathway decreases adenylylcyclase activity, G_i signaling has also been studied by measuring the inhibition of forskolin-stimulated cAMP accumulation (6). However, this assay suffers from a limited dynamic range, since inhibition rarely exceeds 60% of the stimulation. In addition, the assay is expensive, time-consuming, and not well suited for high-throughput screening. Recently, the fluorometric imaging plate reader (FLIPR,[4] Molecular Devices) was developed to perform high-throughput agonist and antagonist screening with

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[4] Abbreviations used: FLIPR, fluorometric imaging plate reader; R-SAT, receptor selection and amplification technology.

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transfected cells in a 96-well microplate format. This robotic system allows rapid screening of compounds present in combinatorial chemical libraries and provides an efficient way to characterize orphan receptors and potential peptide agonists identified by genome sequencing projects. The FLIPR can measure simultaneously in 96 wells of a microplate the increase in intracellular calcium resulting from activation of G_q-signaling pathways in CHO cells. Although activation of G_i-coupled receptors does not normally cause calcium mobilization in CHO cells, mobilization will occur if the receptors are coexpressed with chimeric $G\alpha_q$ proteins containing the five carboxyl-terminal amino acids from $G\alpha_i$ or $G\alpha_o$ (i.e., $G\alpha_{qi5}$ or $G\alpha_{qo5}$) (7) or with full-length $G\alpha_{16}$ (8, 9).

In this report, we have used chimeric G proteins to allow the G_i-coupled opioid receptors to signal through G_q and thus generate a robust calcium response in CHO cells. We then used the FLIPR to measure this response. The chimeric G protein/FLIPR data are consistent with results obtained from the Cytosensor microphysiometer, indicating that artificial G protein coupling does not significantly affect receptor pharmacology. In addition, the FLIPR assay is less expensive and more efficient than traditional assays. We used the chimeric G protein/FLIPR system to screen a panel of opioid peptides and drugs to determine their pharmacologic activities on each of the human opioid receptors. All of the peptides activated at least one receptor, and many activated two or more, supporting a complex model of receptor activation. In addition, this assay was able to identify molecules that behaved as antagonists.

MATERIALS AND METHODS

Cells and culture conditions. Human μ, δ, and κ opioid receptor cDNAs were obtained and used under license from the National Institutes of Health, the University of Arizona, and Temple University, respectively (10–13). The ORL1 cDNA was cloned by reverse transcriptase–polymerase chain reaction (RT–PCR) from human NT neurons (Layton Bioscience, Inc., Atherton, CA) using forward primer 5′-AGGAGGTTGCAGAAGTACC-3′ and reverse primer 5′-CTGTGTGAGCTCTGTGTTGG-3′. The cDNAs encoding $G\alpha_{qo5}$, and $G\alpha_{qi5}$ were described previously (14). The full-length DNA sequences and methods for requesting materials are available in the G Protein Chimera Users Manual posted on our web site (http://gladstone.ucsf.edu/conklin.html). The $G\alpha_{16}$ cDNA was cloned by RT–PCR from TF-1 cells using forward primer 5′-TTTCAGGCAAGGAACTCTAGG-3′ and reverse primer 5′-GTCAAGCAAGGGCAGGAGTAC-3′. Stable CHO-K1 cell lines expressing each receptor were generated by standard methods using G418 selection (GENETICIN, Life Technologies, Gaithersburg, MD). For cotransfection experiments, these stable cell lines were either transiently transfected with one of the three G protein cDNAs or stably transfected with $G\alpha_{qi5}$ using hygromycin B resistance as the selectable marker.

Fluorometric imaging plate reader assay. Cells and ligands were prepared for the FLIPR assay as described (7). For a given experiment, ligands from the same master ligand plate were assayed against all the receptors, eliminating ligand breakdown as the explanation for differential agonist activity across receptors.

Microphysiometry experiments. Cells were prepared for the Cytosensor microphysiometer (Molecular Devices Corp., Sunnyvale, CA) as described (15). To generate dose–response curves, cells were exposed to increasing concentrations of ligand in running medium for 3 min at 30-min intervals, and the effects on acidification rate were measured. The acidification rate was normalized to 100% before the addition of test materials.

Reagents. Peptide hormones were purchased from Peninsula Laboratories (Belmont, CA), and small molecule drugs were purchased from Research Biochemicals International (Natick, MA). Additional supplies of spiradoline were a gift of Pharmacia & Upjohn (Kalamazoo, MI). Butorphanol was purchased from the pharmacy at the San Francisco General Hospital. All other reagents were from Sigma or Fisher Scientific.

RESULTS

Coupling the opioid receptors to G_q. The FLIPR high-throughput system measures changes in intracellular calcium levels that accompany activation of the G_q pathway. To make the normally G_i-coupled opioid receptors activate G_q, we cotransfected each receptor and $G\alpha_{16}$, $G\alpha_{qi5}$, or $G\alpha_{qo5}$ into CHO K1 cells. The transfected CHO cells were then incubated in a medium containing the calcium-sensitive dye Fluo-3 and used in the FLIPR assay to measure receptor-activated increases in intracellular calcium levels. The nociceptin receptor, ORL1, a G_i-coupled receptor closely related to the opioid receptors (10), was also evaluated in this assay. We used the following receptor agonists: DAMGO for the μ opioid receptor (MOR) (11), DPDPE for the δ opioid receptor (DOR) (12), U69593 for the κ opioid receptor (KOR) (13), and nociceptin for ORL1. The MOR and KOR signaled most efficiently when cotransfected with $G\alpha_{qi5}$, and the DOR signaled most efficiently when cotransfected with $G\alpha_{16}$, although it also signaled well with $G\alpha_{qi5}$ (Fig. 1). ORL1 signaled efficiently when cotransfected with $G\alpha_{qi5}$, $G\alpha_{qo5}$, or $G\alpha_{16}$. In the absence of a transfected chimeric G protein, no calcium signal was seen upon activation of any of the receptors. To control for differences in transfection efficiency, equal amounts of DNA encoding each of

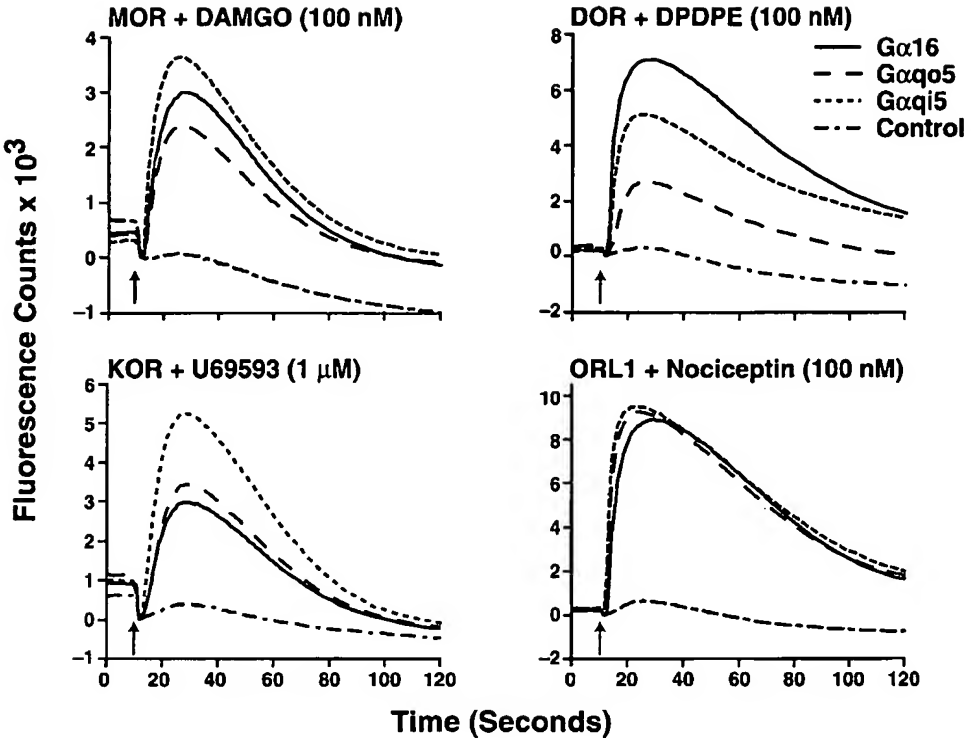

FIG. 1. Preferential coupling of opioid receptors for specific chimeric G proteins. CHO cells stably expressing the indicated opioid receptor were transiently transfected with G protein constructs. Agonist-mediated changes in intracellular calcium were measured by the FLIPR as described under Materials and Methods. DPDPE, DAMGO, and nociceptin were used at 100 nM, and U69593 was used at 1 μM. The arrow indicates the addition of agonist. Each plot represents the mean value of eight determinations from a single experiment. Two additional experiments gave similar results.

the chimeric G proteins were transfected into cells stably expressing one of the receptors. This ensured that, for a given receptor, each transfection produced similar receptor:G protein expression levels (16, 17). This experiment demonstrates that when chimeric G proteins confer G_q-effector coupling to the opioid receptors, receptor activation results in changes in intracellular calcium levels that can be measured by the FLIPR.

Because the chimeric G protein/FLIPR assay relies on artificial G protein coupling to activate the G_q-signaling pathway, we wanted to compare our FLIPR results with those generated from a system that uses endogenous G_i pathways. The Cytosensor microphysiometer can detect changes in extracellular acidification that result from agonist stimulation of G_i-coupled receptors (18). Like the FLIPR, the Cytosensor measures changes in real time, but Cytosensor experiments are more time-consuming and not well suited for high-throughput screening. CHO cell lines stably expressing only the opioid receptors were used for the Cytosensor, and cells stably expressing both an opioid receptor and $G\alpha_{qi5}$ were used in the FLIPR assay. The cells were exposed to limiting dilutions of agonists to generate dose–response curves, which were used to calculate the EC_{50} value for each receptor/agonist pair. In each case, the Cytosensor EC_{50} values were lower than the corresponding FLIPR values (Table 1). This result was not unexpected since similar effects of artificial G protein coupling on EC_{50} values have been noted previously, probably reflecting decreased efficiency of receptor–G protein interaction (14, 19). The fact that the change was not the same for each cell line may reflect differences in the relative amounts of receptor and G protein expressed because these differences are also known to affect EC_{50} values (20). Exposure of parental

TABLE 1

Comparison of EC_{50} Values Calculated from FLIPR and Cytosensor Experiments

Receptor + ligand	FLIPR	Cytosensor	Fold difference
MOR + DAMGO	141 ± 69.3 (2)	9.61 ± 2.78 (5)	15
DOR + DPPPE	23.8 ± 18.5 (3)	1.01 ± 0.16 (6)	24
KOR + U69593	69.6 ± 6.93 (4)	13.4 ± 2.79 (5)	5
ORL1 + nociceptin	8.19 ± 1.19 (6)	0.064 ± 0.0015 (6)	129

Note. Data are expressed (in nM) as geometric means ± 95% confidence interval. The number of experiments used to generate the data is shown in parentheses. For the Cytosensor, drugs were tested in duplicate or quadruplicate at 11 concentrations from 0.01 to 1000 nM (MOR, DOR, and ORL1) or 0.1 to 10,000 nM (KOR). For the FLIPR, drugs were tested in duplicate or quadruplicate at seven concentrations from 0.1 to 100 nM (MOR, DOR, ORL1) or 1 to 1000 nM (KOR).

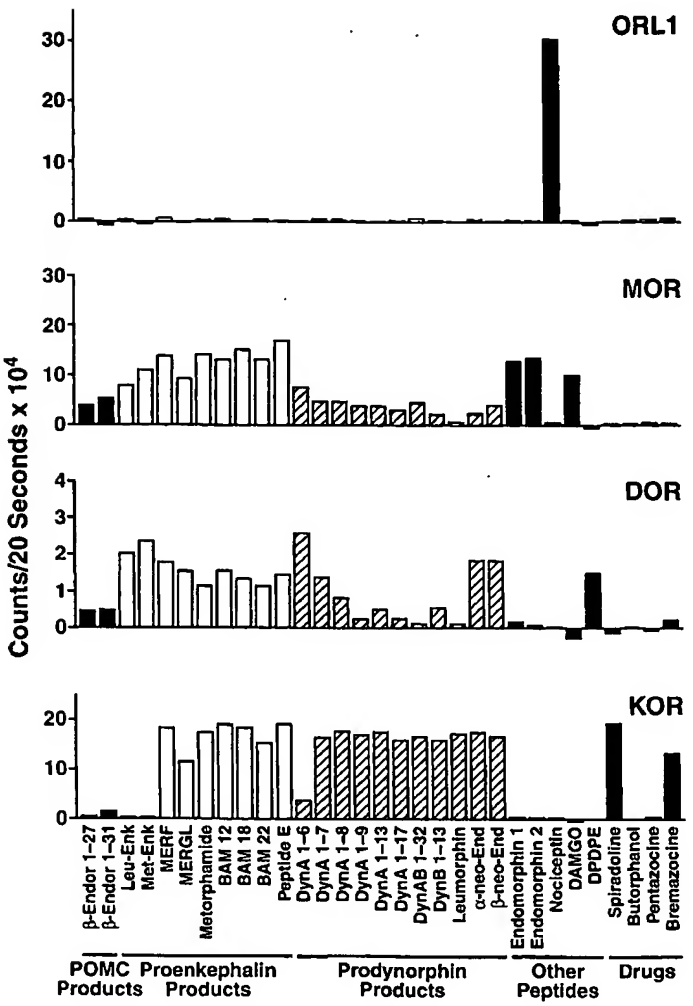

FIG. 2. Agonist activities of 31 peptide and small molecule ligands on the MOR, DOR, KOR, and ORL1. Agonist-mediated changes in intracellular calcium were measured by the FLIPR as described under Materials and Methods. The agonist concentration was 1.0 μM. See Table 2 for peptide ligand abbreviations. Data are expressed as mean of duplicate determinations in a single experiment. An additional experiment gave similar results.

CHO cells to these agonists had no detectable effect on acidification rate or calcium mobilization (data not shown). When combined with the observation that the chimeric G protein/FLIPR assay correctly identified known opioid agonists and antagonists (7) (see Fig. 2), the data presented in Table 1 indicate that artificial coupling of the receptors does not significantly influence receptor pharmacology and validate the use of the assay to identify agonists and antagonists of G_i-coupled receptors.

Cost of operating the FLIPR. The FLIPR is a high-throughput assay that does not require extensive sample handling by the investigator. The cost of labor and reagents to run the FLIPR is estimated to be about $0.40 per data point, and two researchers can generate over 10,000 data points per day (S. MacLennan, Roche Bioscience, personal communication). By comparison, the traditional inhibition of adenylylcyclase assay costs about $1.50 per point, and two researchers can process about 400 data points per day. As a result, the FLIPR assay costs about 67% less per data point and can generate over 25 times more data in a given time period. Other commercially available, 96-well plate-based cyclase assays cost about $4.00 per data point (excluding labor and other reagent costs) and are not automated. Ligand-binding experiments cost about the same as FLIPR experiments, but are less informative because they do not differentiate between agonists and antagonists. GTPase assays have also been used but require the use of radioactivity.

Agonist activity of opioid peptides. Opioid peptides are proteolytically processed from three precursor proteins to yield many different peptides with potential biological activity (21, 22). Although the binding affinities of many of these peptides have been determined (23), they have not been systematically characterized for agonist activity. The chimeric G protein/FLIPR system provided an ideal assay to test the agonist activity of each of the commercially available opioid peptides on each opioid receptor. Figure 2 shows the results of a typical experiment, assaying a panel of 25 naturally occurring peptides (see Table 2), two synthetic peptides, and four synthetic small molecule drugs for agonist activity. These data are interesting for several reasons. First, it is often assumed that peptides derived from each precursor protein act primarily on a specific class of receptor (e.g., enkephalins primarily activate the DOR). Clearly this is not the case, since leu-enkephalin and met-enkephalin potently activated the MOR and DOR, and the other enkephalin peptides activated the MOR, DOR, and KOR. The dynorphin peptides showed strong activation of the KOR, but certain of these peptides also activated the MOR and DOR. Interestingly, dynorphin A(1-7) was a strong KOR agonist, but dynorphin A(1-6), which has one less amino acid, showed very little activity at this receptor. Conversely, dynorphin A(1-6) was among the most efficacious DOR agonists tested, but dynorphin A(1-7) was significantly less effective, and dynorphin A(1-8) even less so. β-Endorphin(1-27) and β-endorphin(1-31) were weakly reactive with the MOR and DOR, and endomorphin-1 and endomorphin-2 were specific for the MOR. The small molecule drug bremazocine showed strong KOR-agonist and weak DOR-agonist activity, but butorphanol and pentazocine did not behave as agonists at any of the receptors in this assay. The control drugs DAMGO, DPDPE, and spiradoline were, as expected, specific for the MOR, DOR, and KOR, respectively. Nociceptin was very specific for ORL1,

TABLE 2

Comparison of Opioid and Other Peptide Sequences

Peptide	Sequence
Proopiomelanocortin (POMC) products	
β-Endorphin (1-27) (β-Endor$_{1-27}$)	YGGFMTSEKSQTPLVLFKNAIIKNAY
β-Endorphin (1-31) (β-Endor$_{1-31}$)	YGGFMTSEKSQTPLVLFKNAIIKNAYKKGE
Proenkephalin products	
Leu-enkephalin (Leu-Enk)	YGGFL
Met-enkephalin (Met-Enk)	YGGFM
Met-enkephalin-Arg-Phe (MERF)	YGGFMRF
Met-enkephalin-Arg-Gly-Leu (MERGL)	YGGFMRGL
Metorphamide	YGGFMRRV-NH2
BAM 12	YGGFMRRVGRPE
BAM 18	YGGFMRRVGRPEWWMDYQ
BAM 22	YGGFMRRVGRPEWWMDYQRYG
Peptide E	YGGFMRRVGRPEWWMDYQRYGGFL
Prodynorphin products	
Dynorphin A(1-6) (DynA$_{1-6}$)	YGGFLR
Dynorphin A(1-7) (DynA$_{1-7}$)	YGGFLRR
Dynorphin A(1-8) (DynA$_{1-8}$)	YGGFLRRI
Dynorphin A(1-9) (DynA$_{1-9}$)	TGGFLRRIR
Dynorphin A(1-13) (DynA$_{1-13}$)	YGGFLRRIRPKLK
Dynorphin A(1-17) (DynA$_{1-17}$)	YGGFLRRIRPKLKWDNQ
Dynorphin AB(1-32) (DynAB$_{1-32}$)	YGGFLRRIRPKLKWDNQKRYGGFLRRQFKVVT
Dynorphin B(1-13) (DynB$_{1-13}$)	YGGFLRRQFKVVT
Leumorphin	YGGFLRRQFKVVTRSQEDPNAYYEELFDV
α-Neoendrophin (α-neo-End)	YGGFLRKYPK
β-Neoendrophin (β-neo-End)	YGGFLRKYP
Other peptides	
Endomorphin-1	YPWF
Endomorphin-2	YPFF
Nociceptin	FGGFTGARKSARKLANQ

Note. Amino acid sequences of peptides derived from the opioid precursor proteins proopiomelanocortin, proenkephalin, and prodynorphin, and other peptides used in thus study, using the single-letter amino acid notation. Abbreviations used in Fig. 2 are shown in parentheses.

and ORL1 did not respond to any of the opioid peptides or small molecule drugs at the highest concentrations tested (1 μM).

Antagonist activity of synthetic drugs. The small molecule drugs butorphanol and pentazocine showed no agonist activity in the chimeric G protein/FLIPR assay at concentrations up to 1 μM, raising the possibility that they might be antagonists or partial agonists. We therefore tested the ability of these compounds to affect signaling activated by MERF, a peptide that activates all three opioid receptors (Fig. 2). MERF was used at 37.5 nM, a concentration below its EC_{50} but high enough to give a detectable signal in the FLIPR assay (Fig. 3). Butorphanol, pentazocine, bremazocine, and the known antagonist naloxone were added at a concentration of 1 μM. Butorphanol antagonized signaling by MERF at each of the opioid receptors, while pentazocine showed antagonist activity at the KOR and MOR, but did not affect MERF activation of the DOR. Bremazocine showed antagonistic activity at the MOR and DOR, but, as expected, also showed potent KOR agonist activity (21, 24). Naloxone was an antagonist at each of the opioid receptors. This experiment demonstrates that antagonists and partial agonists can also be assayed with the chimeric G protein/FLIPR system.

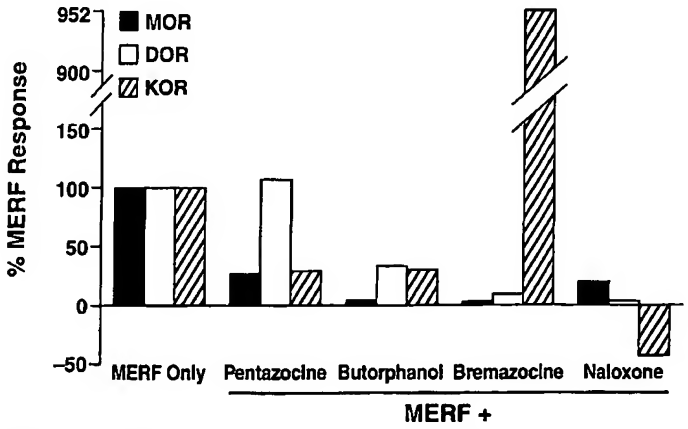

FIG. 3. Antagonist activity of four small molecule drugs on the opioid receptors. Agonist-mediated changes in intracellular calcium were measured by the FLIPR as described under Materials and Methods. MERF was added at 37.5 nM either alone or in combination with the small molecule drugs indicated at 1 μM. Data are expressed as mean of duplicate determinations in a single experiment. Two additional experiments gave similar results.

DISCUSSION

We have used chimeric G proteins to allow G_i-coupled receptors to mobilize intracellular calcium stores in response to agonist stimulation. This response was measured with the FLIPR high-throughput assay system. The chimeric G protein/FLIPR system has several advantages over other assay systems used to characterize G_i-coupled receptors. First, because the experiments reported here use recombinant receptors and G proteins expressed in a well-defined tissue culture cell line, the definition of ligand–receptor interaction is more precise than in studies of whole animals or tissue preparations, where expression of multiple receptor types or family members can be a confounding variable. Second, because the FLIPR assay system measures a physiologic effect (i.e., signaling), the results are more revealing with regard to agonist and antagonist activities than results obtained solely from binding studies, even those using recombinant receptors. Third, because this assay is a stimulation assay, it is better suited for distinguishing subtle differences in agonist potency than the traditional G_i-signaling assay, which measures inhibition of adenylylcyclase and suffers from a limited dynamic range. Fourth, the FLIPR assay is very rapid, taking only 2 min for the fluorometric measurement, and is performed in 96-well plates, making it possible to simultaneously test multiple dilutions of many ligands in duplicate. For example, we were able to test all four receptors with all the ligands described in Fig. 2 in 3 h, including the time to incubate the cells in medium containing the calcium-sensitive dye Fluo-3. Fifth, we estimate that two researchers can generate over 10,000 data points in a day at a cost of about $0.40 per point, making this assay about 25 times more efficient and 67% less expensive than the traditional inhibition of adenylylcyclase assay.

Recently, two other assays have been developed that can measure activation of G_i-coupled receptors in a microplate format. The receptor selection and amplification technology (R-SAT) assay measures increases in β-galactosidase activity associated with receptor-mediated proliferation in NIH 3T3 cells (25). A second assay uses the photoprotein apoaequorin to monitor changes in intracellular calcium levels after receptor activation in CHO cells (9). Both assays use chimeric or "promiscuous" (i.e., $G\alpha_{16}$) G proteins. It is unclear how these assays compare to the chimeric G protein/FLIPR assay in terms of sensitivity and ability to distinguish subtle differences in agonist potency. The fact that the R-SAT assay requires agonist stimulation for many hours may limit its usefulness with unstable, toxic, or scarce ligands.

The FLIPR assay described here relies on chimeric G proteins to alter the signaling specificity of the G_i-coupled opioid receptors to include G_q. In fact, the chimeras are effective for altering the signaling specificity of G_i-, G_s-, and G_q-coupled receptors and have been used to stimulate calcium flux in CHO K1 cells (7); adenylylcyclase in CHO K1 and Cos-7 cells (26, 27); phospholipase C β in CHO K1, HEK 293, and Cos-7 cells (16, 17, 26, 28, 29); Na^+–H^+ exchange in HEK 293 cells (30); NFAT-mediated gene expression in PC12 cells (31); and proliferation in NIH 3T3 cells (25, 32). However, receptors do not always activate the chimeras equally, and some do not activate any at all (26). When we combine our own studies with the reports from other groups, 18 of 20 G_i-coupled receptors efficiently activate chimeric or promiscuous G proteins. (Only the somatostatin and cannabinoid receptors have been reported not to work with the G protein chimeras.) Unfortunately, there is no general rule for predicting whether a particular receptor will work. For receptors that do work, it is important to test multiple chimeras to determine which works most efficiently (see Fig. 1). Since the G protein chimeras have been distributed to over 50 laboratories worldwide, new G_i-coupled receptors are being tested on a regular basis. A continually undated list of references to studies that have used the G protein chimeras is available in the G Protein Chimera Users Manual on our web site (http://gladstone.ucsf.edu/conklin.html).

The data presented in Fig. 2 support a complex model of opioid receptor activation, where a given peptide has different effects on the different receptor family members and peptides differing by as little as one amino acid have different effects on the same receptor. Similarly complex receptor–ligand networks exist for other receptor families, including the serotonin, dopamine, endothelin, orexin, and melatonin receptors (33–36). Why would nature want such a complicated system of ligands and receptors? Presumably this system affords more sophisticated regulation of signaling events. For example, the same concentration of leu-enkephalin maximally activates the DOR, half-maximally activates the MOR, and has little effect on the KOR. Neighboring cells expressing a single type of receptor might then respond differently to equivalent concentrations of this peptide. Likewise, cells expressing more MOR than DOR would respond differently than cells expressing more DOR than MOR. Complex receptor–ligand networks are common in nature and highlight the need for high-throughput assays, like the chimeric G protein/FLIPR assay described here, to easily measure all the potential ligand–receptor combinations. We anticipate that this high-throughput screening assay will be useful not only for accurately defining endogenous ligands, but also for mass screening of potential therapeutic compounds.

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